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Tumor-Associated Macrophages and Microglia:
Double-edged sword in tumor evolvement and invasion

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“Run when you can,
walk if you have to,
crawl if you must;
just never give up.”

(Dean Karnazes)

EIN KLEINER EINBLICK IN DIE KREBSENTSTEHUNG UND DIE ROLLE DER MAKROPHAGEN UND MIKROGLIA

Krebs ist die zweithäufigste Todesursache weltweit und je älter wir werden, desto wahrscheinlicher ist es daran zu erkranken. Diese Krankheit macht vor Niemandem halt; ob Frau, ob Mann, ob alt, ob jung. Jeden kann es treffen und zu jedem Zeitpunkt im Leben. Am Anfang einer Behandlung stellt sich immer die Frage: „Kann ich den Kampf gegen den Krebs gewinnen?“ Und am Ende einer jeden Behandlung bleibt die Unsicherheit: „Habe ich den Kampf gegen den Krebs gewonnen?“ Zu Recht, denn es kann passieren, dass vereinzelte sehr resistente Krebszellen dem Tod entrinnen konnten und auf der Lauer liegen, damit sie später wieder zuschlagen können.....und diesmal stärker als zuvor. Ein Kampf, der zu häufig verloren wird.

Was aber ist Krebs? Einfach beschrieben sind es Zellen, die unkontrolliert wachsen. Was macht diese Krankheit dann so gefährlich?

Fangen wir von vorne an. Der menschliche Körper stellt ein System dar, in dem alle Prozesse nahezu perfekt aufeinander abgestimmt sind. In diesem System gibt es sehr viele verschiedene Kontrollinstanzen, die versichern, dass die Zellen funktionsfähig sind und ihre Aufgaben richtig erfüllen. Sollte das nicht mehr der Fall sein, kann unter anderem ein „Selbsttötungsprogramm“ eingeleitet werden. Dieser programmierte Zelltod ist in jeder Zelle gespeichert, so dass jede Zelle ihn durchlaufen kann, um sicher zu stellen, dass alle Abläufe im Körper weiterhin reibungslos verlaufen. Sei es die Eliminierung von überflüssigen Zellen während der embryonalen Entwicklung, ein Fehler in der DNA durch UV-Strahlung oder einfach nur die Erneuerung von Zellen; die Zelle weiß, wann sie zu gehen hat und Platz schaffen muss für eine neue gesunde Zelle. Die Entstehung einer neuen Zelle wird durch den Reparaturapparat streng kontrolliert, so dass sichergestellt wird, dass die DNA fehlerfrei ist und die Zelle ihre Erlaubnis bekommt zu leben.

Das Problem entsteht, wenn sich zu viele Fehler in der DNA summieren, die verschiedenen Kontrollinstanzen diese Fehler übersehen und sogar selber Defekte aufweisen. Das ist der Zeitpunkt, wo die Zellen dem Stoppsignal zum unkontrollierten Wachstum und sogar dem Selbsttötungsprogramm entgehen. Es bildet sich eine wuchernde und immer größer werdende Zellmasse, die sich unendlich oft teilen kann und unsterblich zu sein scheint. Diese Zellen schmeißen die ganzen kontrolliert ablaufenden Prozesse über einen Haufen....wachsen und

wachsen. Wenn die Zellmasse eine bestimmte Größe erreicht hat, braucht sie die Neubildung von Blutgefäßen um weiter wachsen zu können. Wie sonst soll sie Sauerstoff und Nährstoffe bekommen, die sie zum Überleben braucht? Wachstumsfaktoren werden im Übermaß ausgeschüttet, welche dazu führen, dass die neugebildeten Blutgefäße im Übermaß und unorganisiert wachsen. Diese Blutgefäße sind gekennzeichnet durch undichte Stellen und eine schlechte Durchblutung. Wenn nun diese Zellen in benachbartes Gewebe eindringen und durch die undichten Stellen in den Blutgefäßen zu entfernten Organen gelangen, werden sie zu bösartigen Krebszellen. Diese breiten sie sich unkontrolliert im Körper aus, sie streuen, und die Entstehung von neuen unkontrolliert wachsenden Zellmassen beginnt, den Metastasen. Krebs wird zu einer lebensbedrohlichen Krankheit.

Doch wo bleibt das Immunsystem? Das Immunsystem ist eins der elementarsten Funktionen im menschlichen Körper, das sehr komplex und auf einander abgestimmt ist. Es schützt uns vor gefährlichen Eindringlingen und stellt sicher, dass eigene entartete Zellen zerstört werden. Diese Aufgaben verlangen das Zusammenspiel von vielen verschiedenen Mitgliedern, wozu auch die Immunzellen zählen. Diese Immunzellen sind auch als weiße Blutkörperchen bekannt und unter ihnen befinden sich die Makrophagen. Makrophagen zirkulieren in unserem Körper und suchen die Umgebung nach Fremdkörpern ab. Treffen sie diese an, fressen sie die Fremdkörper auf, kommunizieren durch das Aussenden von vielen verschiedenen Stoffen mit ihrer Umgebung und suchen den Kontakt zu anderen Immunzellen auf. Diese und noch viele weitere initiierte Prozesse sorgen dafür, dass die Gefahr eliminiert wird. Darüber hinaus spielen sie auch eine wichtige Rolle in anderen Bereichen, die zum Erhalt des Gleichgewichts der Körperfunktionen beitragen. Diese sind unter anderem die Wundheilung und Reparatur von Gewebe, wo auch das Wachstum von neuen Blutgefäßen benötigt wird. Eine Fähigkeit, die Tumorzellen ausnutzen. Makrophagen befinden sich in allen Organen und selbst im Gehirn findet man sie. Dort sind sie als Mikroglia bekannt. Mikroglia erfüllen ganz spezielle und auf das Nervensystem abgestimmte Aufgaben.

Makrophagen und Mikroglia, die den Tumor infiltrieren, nennt man auch Tumor-assoziierte Makrophagen und Mikroglia (TAMMs). Je nachdem welche Signale sie aus ihrer Umgebung bekommen, können sie ein bestimmtes Erscheinungsbild annehmen. Durch ihre Plastizität können sie auch schnell zwischen unterschiedlichen Aktivierungszuständen wechseln und so viele verschiedene Aufgaben durchführen. Manche davon behindern das Krebswachstum und seine Streuung, sie sind anti-tumoral; und andere fördern diese Prozesse, sie sind pro-tumoral.

In den meisten Fällen eliminiert das Immunsystem (Makrophagen und Mikroglia sind hier mit eingeschlossen) entartete Krebszellen, die immer wieder versuchen das Immunsystem zu überlisten. Makrophagen und Mikroglia zeigen ein anti-tumorales Erscheinungsbild. Viele potenzielle Krebszellen fallen dem Immunsystem zum Opfer, jedoch nicht alle. Irgendwann schaffen es die Krebszellen das Immunsystem auszutricksen. Sie bilden Moleküle auf ihrer Oberfläche ab, die das Signal „Iss mich nicht“ vermitteln und senden Stoffe aus, die den Zerstörungsmodus der Immunzellen unterdrücken. Aus Feinden werden Verbündete. An Stelle den Krebs zu bekämpfen, helfen ihm nun Makrophagen und Mikroglia bei der Entstehung, beim Wachstum und bei der Streuung; sie sind pro-tumoral. Viele dieser Mechanismen sind schon erforscht, aber viele andere warten darauf ergründet zu werden. Das ist jedoch leichter gesagt als getan. Krebs ist eine sehr komplexe Krankheit, die in jedem Organ etwas anders aussieht und die wir bis zum heutigen Tag immer noch nicht komplett verstanden haben. Eine Krankheit, in der sich Makrophagen und Mikroglia als zweischneidiges Schwert erweisen können.

Heutzutage gibt es viele verschiedene Therapiemöglichkeiten und es werden immer mehr entwickelt. Und doch verlieren wir zu oft den Kampf gegen den Krebs. Es scheint zu viele Hürden zu geben, die es zu überwinden gibt. Man muss z.B. die Medikamente erfolgreich zu den Krebszellen befördern, obwohl die Blutgefäße um den Krebs herum durchlässig sind. Dann kommt hinzu, dass die Verabreichung eines Medikaments Krebszellen abtötet, die eine spezielle Mutation aufweisen. Jedoch bilden Krebszellen eine Resistenz gegenüber dem Medikament auf und es gibt genügend andere Krebszellen, die andere Mutationen besitzen. Und dann ist da noch das Immunsystem. Es gibt sehr viele verschiedene Ansätze das Immunsystem wieder „zurück zu programmieren“, so auch für die Makrophagen und Mikroglia. Diese Zellen so zu manipulieren, dass sie wieder anti-tumoral agieren. Eine sehr wichtige Erkenntnis bei alledem ist, dass eine Behandlungsmöglichkeit oft nicht ausreicht. Eine Kombination mit Einbezug des Immunsystems scheint vielversprechend zu sein. Jedoch ist der Weg zur Krebsbekämpfung noch lang. Um irgendwann den vollen Umfang an Behandlungsmöglichkeiten in der Krebstherapie nutzen zu können, brauchen wir das Wissen über jeden einzelnen Ablauf, der in Verbindung mit der Krebsentstehung, dem Krebswachstum und der Streuung steht. Dazu gehört auch die „Fehlfunktion“ des Immunsystems dem Krebs als Freund und nicht als Feind zu begegnen. Ich hoffe, dass ich mit meiner Doktorarbeit einen kleinen Teil dazu beigetragen habe, diese Krankheit besser zu verstehen, insbesondere welche Rolle Makrophagen und Mikroglia darin spielen können.

ABSTRACT

Tumor-associated macrophages (TAMs) are among the most abundant cell types in the tumor microenvironment. TAM infiltration is usually linked to tumor progression, metastasis and poor clinical outcome in most human cancers. These cells sense a wide range of intra- and extracellular signals, such as chemokines and growth factors but also disturbances in pH or availability of oxygen. Due to their remarkable plasticity, TAMs can react immediately to these signals and acquire or switch to different phenotypes and activation states accordingly. For the past decade, TAMs have been recognized as a new therapeutic target. Even though diverse monotherapy strategies targeting TAMs have shown limited success, there are many different experimental studies that have shown promising results when approaching TAM subsets in combination with several other treatments, such as chemotherapies. However, our knowledge about the full scope of TAM heterogeneity and function in tumor evolvement and invasion is still lacking.

In trying to improve the understanding about the versatile role of TAMs during tumor progression and invasion, several contributions have been made in this thesis. First, we show a novel mechanism whereby the TAM population can be skewed to contain mainly anti-tumoral M1-like Macrophages. Thus, we show that the overexpression of Semaphorin (SEMA)3A by tumor cells selectively induced the proliferation of M1-like macrophages and decreased the expansion of M2-like macrophages. This resulted in enhanced recruitment and activation of cytotoxic $CD8^+$ T lymphocytes and NK cells which in turn inhibited tumor growth. Second, we demonstrate that macrophage-derived Vascular Endothelial Growth Factor (VEGF)-C improved vessel functionality and thereby decreased pulmonary metastasis while tumor-derived VEGF-C increased vessel abnormalization and lung metastasis. Third, we elucidated that Cripto-1 vaccination decreased lung metastasis by inducing a humoral response leading to activated NK cell killing. Finally, we identified that microglia (resident macrophages of the brain) but not bone marrow-derived macrophages (BMDMs) induced Platelet-derived growth factor receptor (PDGFR)B expression on glioma cells and thereby enhanced their migratory capacity.

LIST OF SCIENTIFIC PAPERS

I. Wallerius M*, **Wallmann T***, Bartish M, Östling J, Mezheyeuski A, Tobin NP, Nygren E, Pangigadde P, Pellegrini P, Squadrito ML, Pontén F, Hartman J, Bergh J, De Milito A1, De Palma M, Östman A, Andersson J, Rolny C. **Guidance Molecule SEMA3A Restricts Tumor Growth by Differently Regulating the Proliferation of Tumor-Associated Macrophages**
Cancer Res. 2016 Jun 1;76(11):3166-78

II. **Wallmann T**, Landwehr LS, Squadrito ML, Wallerius M, Agardy D, Bartish M, Hartman J, Bergh J, De Palma M, Rolny C. **Macrophage-derived Vascular Endothelial Growth Factor C decreases hematogeneous metastatic dissemination by normalizing the tumor vasculature**
Manuscript

III. Witt K*, Ligtenberg MA*, Conti L, Lanzardo S, Ruiu R, **Wallmann T**, Tufvesson-Stiller H, Rolny C, Lladser A, Lundqvist A, Cavallo F, Kiessling R. **Cripto-1 plasmid DNA vaccination targets metastasis and cancer stem cells in murine mammary carcinoma**
Cancer Immunol Res. 2018 Aug 24; in press

IV. **Wallmann T**, Zhang XM, Wallerius M, Bolin S, Joly AL, Sobocki C, Leiss L, Jiang Y, Bergh J, Holland EC, Enger P, Andersson J, Swartling F, Miletic H, Uhrbom L, Harris RA, Rolny C. **Microglia induce PDGFRB expression in glioma cells to enhance their migratory capacity**
Accepted, iScience

** equal contribution*

CONTENTS

LIST OF ABBREVIATIONS

α-SMA	α-smooth muscle actin
bFGF	basic fibroblast growth factor
BMDM	Bone marrow-derived macrophage
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster Differentiation
CNS	Central nervous system
COX2	Cytochrome c oxidase subunit 2
CSC	Cancer stem cell
CSF1	Colony stimulating factor 1
CSF1R	Colony stimulating factor 1 receptor
CX_3CR1	CX_3C chemokine receptor 1
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
GAM	Glioma-associated microglia
GBM	Glioblastoma multiforme
GDNF	Glial-derived neurotrophic factor
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HSC	Hematopoietic stem cell
IBA1	Ionized calcium-binding adapter molecule 1
IDH	isocitrate dehydrogenase [NADP]
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
IRF	Interferon regulatory factor
LEC	Lymphatic endothelial cell
MHC	Major histocompatibility complex

MMP	Matrix metalloproteinase
MMTV-PyMT	Mouse mammary tumor virus-polyoma middle T antigen
MRC	Mannose receptor C-type
NK cell	Natural killer cell
NO	Nitric oxide
NP	Neuropilin
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
ROS	Reactive oxygen species
Sall	Sal-like
SEMA	Semaphorin
TAM	Tumor-associated macrophage
TAMM	Tumor-associated macrophage and microglia
TGF	Transforming growth factor
TNF	Tumor necrosis factor
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 INTRODUCTION

Prior to 2011, there has been limited attention to the role of the tumor microenvironment in tumorigenesis. In 2000, Hanahan and Weinberg introduced six hallmarks of cancer that underlined the sequential progression of cells to cancer cells during multiple genetic alterations, which by then only involved the tumor cells (1). However, during the last decades it has become more and more evident that the tumor microenvironment plays an important role. Therefore, in 2011, Hanahan and Weinberg revised the hallmarks of cancer and added four additional hallmarks that include cancer-related inflammation and the active participation of tumor-associated stromal cells in cancer development (2).

1.1 BREAST CANCER

Breast cancer is the second most common type of cancer worldwide and the most common among women (3, 4). This disease is very heterogeneous and needs to be characterized precisely to choose the best treatment options (5). The most useful and important characteristics to predict prognosis and responsiveness to treatment have been proven to be the anatomical and histological determination (6). Anatomical staging is defined by the TNM categories, which are the tumor size (T), lymph node involvement (N) and the existence of metastases (M) (7). Histological features include the grading containing information about cell proliferation and the receptor status. Based on the receptor expression, breast cancer has been divided into four distinct molecular subtypes. These are luminal A, luminal B, HER2-enriched and basal-like breast cancers (**Figure 1**) (8).

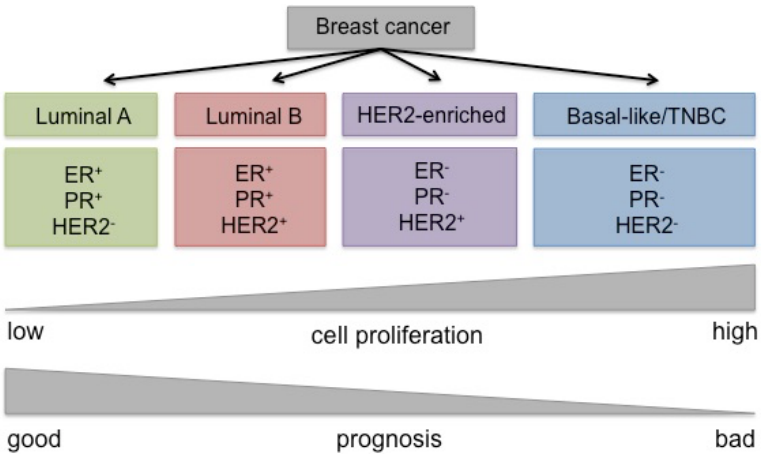

Figure 1. Molecular subtypes of breast cancer.

Basal-like breast cancers overlap with triple-negative breast cancers (TNBC) but it is not the same disease. In fact, 20% of TNBC are not basal-like (9). TNBC resembles the most aggressive subtype of invasive ductal carcinoma, accounts for around 15-20% of breast cancer incidences and is associated with poor prognosis (10-12). TNBC shows higher metastatic potential compared to other subtypes and has the main metastatic sites in bone, lung, liver and brain (13, 14). Lymph node metastasis is observed less frequently but it is a crucial prognostic factor (15, 16). Breast cancer treatment is chosen based on the parameters described above and includes surgery, neoadjuvant and adjuvant therapy treatments (4).

1.2 GLIOMA

Gliomas are the most common primary tumors of the central nervous system (CNS) accounting for almost 80% of all malignant primary tumors (17). They include astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas with astrocytoma being the most common type of gliomas (18). Gliomas can be classified into grades based on malignancy (I-IV), where grade IV is the most malignant, undifferentiated and invasive occurring type and denoted glioblastoma multiforme (GBM) (19). GBMs can arise from primary or secondary astrocytomas and together with diffuse (grade II) and anaplastic (grade III) astrocytomas most often occur in cerebral hemispheres (20). Primary GBMs arise *de novo*, while secondary GBMs progress slowly from low-grade astrocytomas (19). Grade II and III astrocytomas generally harbor a mutation in the cytoplasmic isocitrate dehydrogenase [NADP] (IDH)1, while GBMs are mostly IDH1 wildtype (20). Based on gene-expression analyses gliomas can be further subdivided into classical, mesenchymal, neural and proneural subtypes (**Figure 2**) (21). Low-grade gliomas are mostly of a neural and proneural subtype, while the classical and mesenchymal subtypes occur more frequently in high-grade gliomas (21, 22). The growth pattern of gliomas is characterized by tumor extensions and tumor microsatellites growing far away from the main tumor mass into the normal brain (23). Conventional therapy for GBMs such as surgery, radiation and chemotherapy prolongs survival but is not curative (24).

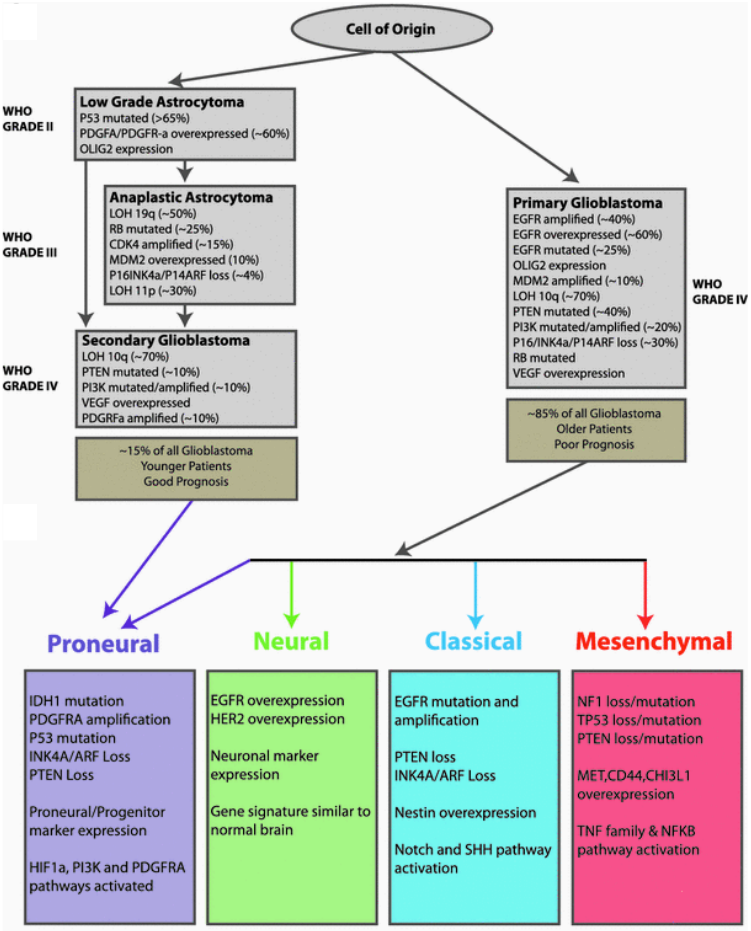

Figure 2. Molecular subtypes of astrocytomas. Adapted from (18).

1.3 THE IMMUNE SYSTEM

In a living organism, one of the immune system's primary functions is to resist pathogens. This host defense system is composed of the innate and adaptive immune systems. These subsystems complement each other and are highly balanced. The interplay between the innate and adaptive immune systems is essential to ensure long-lasting protection against infections and exhibits high complexity (25).

The innate immune system consists of the complement system, soluble factors such as cytokines, natural killer (NK) cells and phagocytic cells such as macrophages and dendritic cells (DCs) (26). Innate immunity enables the body to respond immediately against invading microorganisms and provides most of the every day protection. Since this protection is incomplete, the body requires adaptive immunity, which is a more sophisticated system. Adaptive immunity is tailor-made and antigen-specific against each intruder. Cells of the

adaptive immune system, B and T lymphocytes, possess unique antigen receptors, which enable them to efficiently eradicate pathogens. Although the adaptive immune response to primary infection by a pathogen is slower, the response is more powerful when the pathogen is encountered the second time due to long-lived memory B and T lymphocytes (27). This two-component immune system shows a complicated and fine-tuned network evolved to clear infections efficiently and to protect the host.

1.4 MACROPHAGES AND MICROGLIA

Macrophages are leukocytes that are found in all tissues in differing abundances performing a variety of functions within and between distinct tissues. Although macrophages are mainly known for their phagocytic capacity, they are also involved in many other important processes that provide homeostasis, inflammation and immunity. In the different tissues, macrophages exert substantial functional specialization (28). Macrophages are highly plastic cells that adapt quickly to microenvironmental stimuli and have been shown to be able to respond effectively to environmental changes (29, 30).

In the brain, microglia are a unique population of long-lived tissue-resident cells that share numerous macrophage markers, such as colony stimulating factor 1 receptor (CSF1R), ionized calcium binding adaptor molecule (IBA)1 and F4/80 (31). As macrophages, microglia survey their surroundings and, if needed, phagocyte debris, apoptotic cells and pathogens. Microglia and macrophages regulate migration, differentiation and survival of different cell types. Depending on the activation signal they receive, macrophages and microglia produce a number of anti- and pro-inflammatory cytokines and growth factors (32). However, microglia differ in several aspects such as origin and gene transcription. Transcriptome analyses have revealed over 200 genes, such as Sal-like (Sall)1 and purigenic G-protein coupled P2y12 receptor, and several microRNAs that are uniquely expressed by microglia (31). This might not seem surprising since the brain has a unique microenvironment, where microglia exert very specific and distinct functions to maintain CNS integrity. Through these functions, microglia support processes such as neurogenesis, neuronal differentiation, migration and maintenance (33-35).

1.4.1 Origin and Maintenance

The origin of macrophages and microglia has been discussed for a long time. Lineage-tracing experiments in mice revealed that macrophages and microglia originate from diverse lineages. During embryonic development, macrophages derive first from the yolk sac followed by hematopoiesis of circulating monocytes in the fetal liver. After birth, hematopoiesis in the liver is replaced by bone marrow hematopoiesis, which is by then the major source of circulating monocytes (36). However, not all tissue-resident macrophages derive from circulating monocytes and persist in adulthood, as the skin, pancreas, liver, spleen and brain are populated by yolk sac progenitors, while in the kidneys and lung they have a chimaeric origin arising from yolk sac and bone marrow (**Figure 3**) (37, 38).

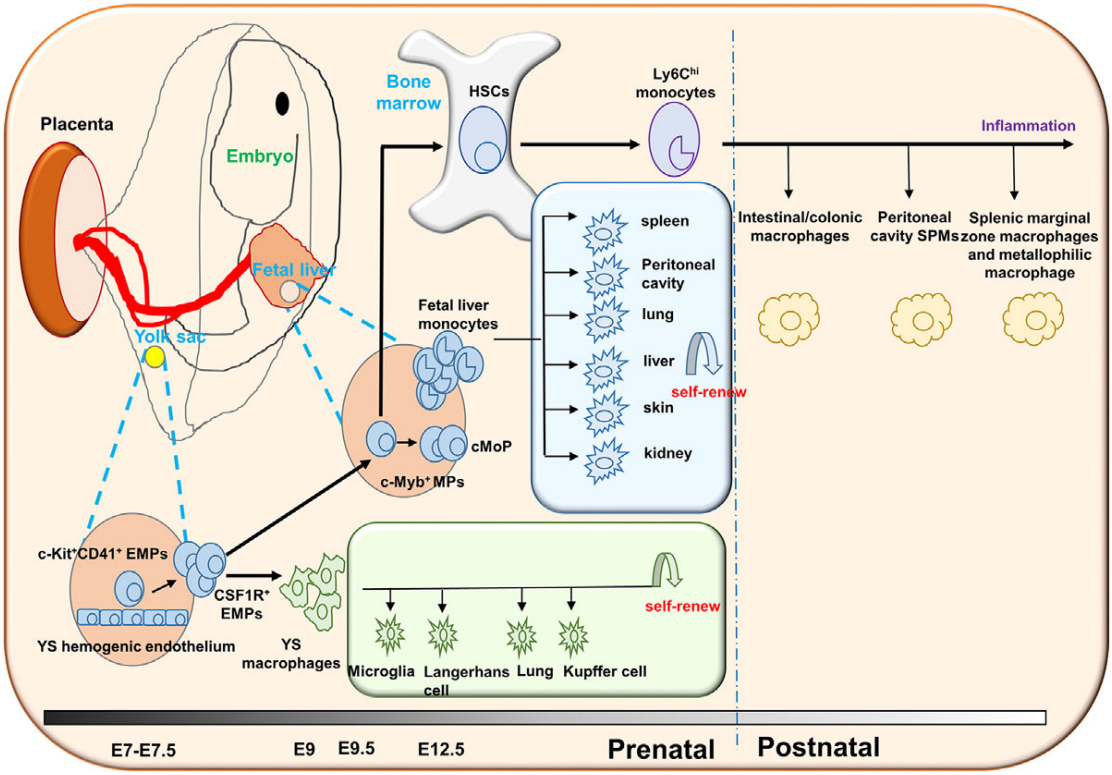

Figure 3. Origin and ontogeny of tissue-resident macrophages and microglia. Adapted from (38).

It is important to consider that both microglia and macrophages may reside in the CNS but develop independently from each other. In mice, at the eighth day after conception $CD45^-$ $c\text{-}kit^+$ erythromyeloid precursors are the earliest yolk sac progenitors that can give rise to microglia. Microgliogenesis is dependent on transcriptions factors Pu.1 and Interferon regulatory factor (Irf)8, and is modulated by matrix metalloproteinases (MMP)-8 and -9 (39). Meningeal macrophages derive from hematopoietic stem cells (HSCs) and turn over rapidly

(38). In comparison, microglia survive and renew themselves throughout lifetime without any influx of circulating monocytes under physiological conditions (40). Thus, depending on the tissue, the maintenance of tissue-specific macrophages and microglia ranges between being exclusively embryo-derived, like microglia in the brain, to being constantly replaced by bone marrow-derived macrophages (BMDMs), such as *lamina propria* macrophages, found in the intestine (**Figure 3**) (41, 42). Interestingly, monocytes that infiltrate tissues to replace embryonic macrophages by hematopoiesis, as observed in the heart, can adapt and differentiate into resident macrophages. This finding underlines that macrophages, independent of the origin, can exert similar functions when they receive tissue-specific signals (43).

1.4.2 Classification and Activation

The classification of macrophages and microglia is challenging and many aspects have to be taken into account when describing distinct subtypes. It is important to consider the source of macrophages and microglia, the system used and the characterization of these cells using many different markers. Based on a framework including the phenotype, physiological activation and functional activity, macrophages have been classified into two subsets, the 'classically activated' M1 macrophages and the 'alternatively activated' M2 macrophages (44).

Macrophages independent of origin

In the presence of pathogens, necrosis or pro-inflammatory molecules such as interferon (IFN)-y or tumor necrosis factor (TNF)-α, macrophages adopt the M1-like phenotype. They release pro-inflammatory cytokines and chemokines (e.g. interleukin (IL)-1, IL-23, chemokine (C-C motif) ligand (CCL)8), are angiostatic, produce cytotoxic mediators, such as reactive oxygen species (ROS), and are involved in T helper (T_H)1-mediated immune response (45-48). Their phagocytic capacity allows them to clear the body of debris, pathogens and cancer cells. Moreover, they express high levels of major histocompatibility complex (MHC) class II molecules that activate the adaptive immune system and recruit T lymphocytes by antigen presentation (49, 50). On the other hand, macrophages that respond to T_H2 cytokines (IL-4, IL-13) acquire the M2-like phenotype. Thereby they down-regulate their pro-inflammatory activities and instead produce anti-inflammatory mediators such as IL-10 and transforming growth factor (TGF)-β (51). M2-like macrophages harbor also high phagocytic capacity as they are involved in fibrosis, tissue repair and wound healing and

promote angiogenesis (52). Even if macrophages have acquired a specific phenotype, due to their remarkable plasticity, they react quickly to microenvironmental stimuli and switch between the different phenotypes to execute the tasks needed (**Figure 4**) (49).

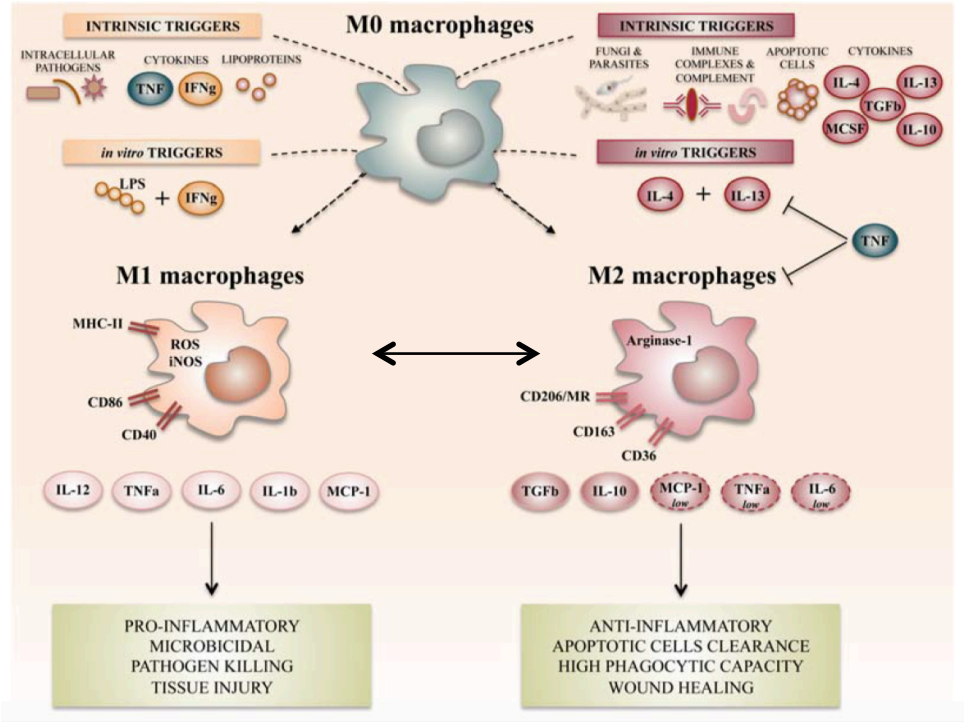

Figure 4. Summary of the main macrophage polarization states of activated macrophages. Adapted and modified from (53).

Microglia

Under homeostatic conditions microglia are known as 'resting' or 'surveillant' microglia that actively screen the CNS to maintain physiological functions (54). As soon as microglia encounter disturbances they become activated and adopt either a cytotoxic or neuroprotective profile depending on the signals they receive (55). Microglia have been divided into states of 'classical activation', 'alternative activation' and 'acquired deactivation' similar to that of extracranial macrophages (**Figure 5**) (56). In short, classical activated microglia respond to injury and infection producing pro-inflammatory cytokines such as IL-1β, TNF-α, nitric oxide (NO), ROS and proteases (57-59). Thereby they upregulate CD11b and IBA1 and start to express genes that are associated with antigen presentation such as MHC class II, cluster differentiation (CD)80 and CD86, resembling an M1-like phenotype of extracranial macrophages (60). Alternatively activated M2-like microglia respond to and release the cytokines IL-4 and IL-13 to promote anti-inflammation, tissue repair and the uptake of debris by upregulating scavenger receptors, such as Mannose receptor C-type (MRC)1, similarly to

extracranial macrophages (46, 61-63). Importantly, M2-microglia support neuron survival by releasing insulin-like growth factor (IGF)-1 (64).

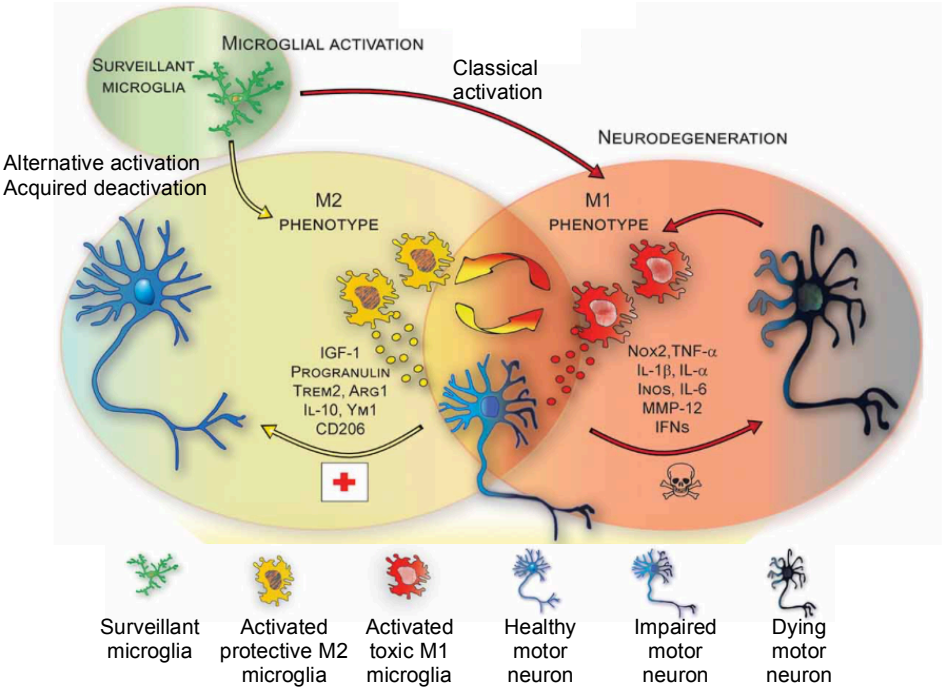

Figure 5. Summary of the main microglia polarization states. Adapted and modified from (65).

As macrophages, microglia are very plastic cells that can switch between different phenotypes (66). Furthermore, the binary classification of macrophages and microglia into M1- and M2-like phenotypes is not as simplistic as described and includes many fine-tuned subtypes. For instance, macrophages and microglia can show expression of both M1- and M2-like markers that can be differently balanced depending on the tissue, signal and function (66, 67).

1.5 TUMOR IMMUNOLOGY

It is thought that the first immune response at the early stage of neoplastic progression resembles the response to acute tissue injury resulting in the infiltration of inflammatory cells (68). If the immune cells fail to eradicate the neoplasm, features of acute inflammation shift to features of chronic inflammation within the local tissue that fosters cancer development. During the persistent recruitment of immune cells, tumor cells secrete different soluble factors that alter the microenvironment and recruit additional myeloid cells and lymphocytes (69, 70). Once the immune cells arrive at the tumor site, they are exposed to many different

tumor secreted factors (e.g. CCL2, IL-4, IL-10, cytochrome c oxidase subunit (COX)2) that skew immune cells from an anti-tumoral phenotype to a pro-tumoral phenotype, so that tumor cells can evade immune destruction (71). As a consequence, these pro-tumoral immune cells release various molecules like growth factors, angiogenic factors, cytokines and proteases that promote tumor growth, invasion and metastatic dissemination (72-74).

1.5.1 Tumor microenvironment

The tumor resembles an autonomous organ that consists of many different components including tumor cells themselves, extracellular matrix (ECM), blood and lymphatic vessels and immune-inflammatory cells that represent the tumor microenvironment (TME) (75). The release of cytokines, growth factors and hormones by the different players adds another layer of complexity in a very dynamic network during tumor progression and metastasis (**Figure 6**). The number and heterogeneity of all factors varies in different tumor and cell types and therefore it is very important to consider these diversities in anti-cancer therapy (76, 77).

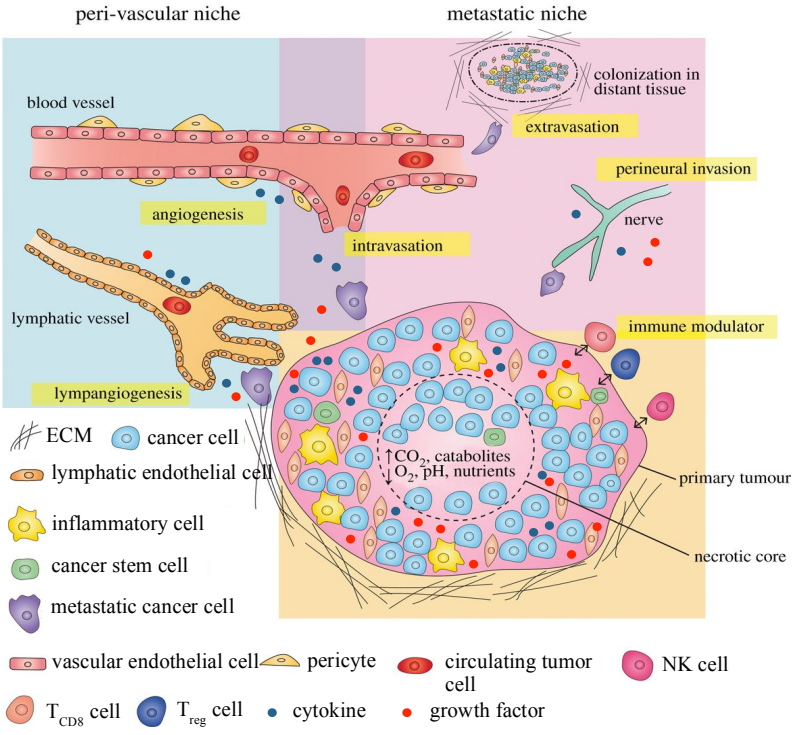

Figure 6. Tumor microenvironment of solid tumors. In the stroma different types of cells and molecules contribute to tumor progression and metastasis. Adapted and modified from (78).

1.6 TUMOR-ASSOCIATED MACROPHAGES AND MICROGLIA (TAMMS)

In the tumor microenvironment macrophages and microglia are known as tumor-associated macrophages and microglia (TAMMs) deriving from tissue-resident macrophages or microglia and from circulating monocytes of the peripheral blood (79). They infiltrate the tumor stroma and can represent the most abundant cell type with more than 50% of the tumor mass (80). In the majority of cancer types, except for prostate, gastric and colon cancer, high infiltration of TAMMs is associated with lower patient survival (81).

Macrophages

Tumor-associated macrophages (TAMs) are major contributors to cancer-related inflammation and in a tumor TAMs consist of a heterogeneous population showing both M1- and M2-like phenotypes (as described above). During cancer initiation, accumulation of M1-like TAMs contributes to cancer elimination, known as immune surveillance. The accumulation of M1-like TAMs activates cytotoxic lymphocytes and thereby restricts tumor growth. However, tumor cells can escape the immune surveillance and secrete immunosuppressive factors skewing immune cells from an anti-tumoral to a pro-tumoral phenotype (82). Hence, the proportion and abundance of TAMs shifts toward an M2-like phenotype during tumor development (83, 84). This is important for tumor progression and metastasis as M2-like TAMs express high levels of angiogenic factors, anti-inflammatory cytokines and scavenging receptors (85). During the course of tumor growth, TAMs constantly undergo phenotypic changes in response to microenvironmental factors (86). In fact, depending on the niche, TAMs express different molecules and exert distinct functions. For instance, TAMs around blood vessels release pro-angiogenic factors like Vascular Endothelial Growth Factor (VEGF)-A to induce tumor angiogenesis, while TAMs at the invasive front produce MMP-2 and -9 to degrade ECM proteins and to facilitate tumor dissemination (87).

As mentioned earlier the M1/M2 polarization model is a very simplistic view on macrophage phenotypes. In 2014, transcriptome-based network analysis on human macrophages revealed a spectrum of different phenotypes upon stimulation of macrophages with diverse activation signals (88). Furthermore, the comparison of normal renal tissue with patient samples of clear cell renal cell carcinoma using single-cell analysis via mass cytometry identified 17 TAM phenotypes. In fact, our group performed single cell sequencing analyses of tumor-associated macrophages from a mouse mammary tumor virus-polyoma middle T antigen (MMTV-PyMT) tumor model and uncovered 9 different subgroups based on the transcriptome.

Intriguingly, these phenotypes change dynamically during tumor progression (unpublished data).

Microglia

Glioma-associated microglia (GAMs) promote glioma growth, invasion, immunosuppression and angiogenesis by releasing cytokines, growth factors and enzymes similar to that of extracranial macrophages. Microglia within a brain tumor mass show also both M1- and M2-like phenotypes with the M2-like phenotype becoming the predominant one during tumor progression. In glioma patients, high infiltration of $CD163^{+}$ and $CD204^{+}$ (human M2-markers) microglia are associated to poor clinical prognosis (89, 90). The inhibition of CSF1R re-educates GAMs from an M2-like phenotype to an M1-like phenotype enhancing their phagocytic capacity and impairing tumor-promoting functions (91).

Even though TAMMs share many characteristics, it is also important to understand the differences, since the brain displays a unique microenvironment with special needs. Unless a neuropathological condition occurs, it is microglia that reside almost exclusively in the brain. The disruption of the blood brain barrier allows the infiltration of monocytes from the periphery (92). Comparing the expression profiles of TAMMs from glioma-bearing mice with naïve microglia, RNA microarray analysis has revealed a different expression profile for around 1,000 transcripts. These TAMMs showed only partial overlap in their expression pattern with known signatures from *in vitro* polarized M1- and M2-like macrophage subtypes (93).

1.6.1 Recruitment

As discussed before, tissue-resident macrophages can arise from distinct populations and monocytes can comprise one of this population. The CSF1/CSF1R axis plays an important role in monopoiesis and mice deficient in either one of the factors show decreased numbers of monocytes in the BM and circulation (94, 95). Immature monocytes are $Ly6C^{hi}$ and become upon maturation $Ly6C^{lo}$, which can occur either in the BM or circulation (96, 97). Blood monocytes can be divided into two functional subsets, based on the expression of different markers, namely short-lived inflammatory CX_3C chemokine $receptor1^{lo}$Chemokine (C-C motif) $receptor2^{+}$ ($CX_3CR1^{lo}CCR2^{+}$)$Ly6C^{hi}$ monocytes that circulate and are recruited to inflamed tissues and long-lived resident $CX_3CR1^{hi}CCR2^{-}Ly6C^{lo}$ monocytes. These $CX_3CR1^{hi}CCR2^{-}Ly6C^{lo}$ monocytes are the precursors for myeloid cells in the tissues under

homeostatic conditions and their development is dependent on the orphan nuclear hormone receptor transcription factor *Nr4a1* (98, 99). Expression of G-coupled receptor for sphingosine-1-phosphate (S1PR5) induces the release of patrolling Ly6C^{lo} monocytes from the BM (100). These patrolling monocytes crawl along blood vessel walls scanning for damage and the presence of pathogens (99).

Under inflammatory conditions, such as during tumor growth, CCL2 is upregulated and recruits Ly6C^{hi} monocytes. These inflammatory monocytes express MHC class II and are good antigen-presenting cells (101, 102). However, during tumor progression Ly6C^{hi} monocytes can be the precursors for TAM subsets and differentiate into Ly6C^{int} that give rise to MHC class II^{lo} and MHC class II^{hi} TAMs. Characterization of these two subsets revealed that MHC class II^{lo} TAMs resemble the M2-like phenotype, are proangiogenic, enriched in hypoxia and efficiently suppress T cell proliferation. On the contrary, MHC class II^{hi} TAMs express a repertoire of genes that resembles more an M1-like phenotype and are mainly found in normoxic regions (103). The infiltration, differentiation and proliferation of tumor-infiltrating Ly6C^{hi} monocytes is also dependent on CSF1R signaling since blockage of this signaling pathway impairs these functions (104). Indeed, high levels of CSF1 are correlated with macrophage infiltration in human metastatic breast cancer (105). Homozygous null mutation of CSF1 in a mouse model of breast cancer reduced TAM infiltration and abolished tumor progression and dissemination. Overexpression, on the other hand, increased tumor growth and metastasis (106). Moreover, inhibition of the CSF1R did not only abrogate TAM infiltration but also increased the recruitment of CD8$^+$ T cells resulting in reduced mammary and cervical tumor growth (107).

Microglia

Glioma cells secrete several cytokines and chemokines to recruit microglia into the tumor mass. For instance, CCL2 is also a major contributor to microglia infiltration as well as CX_3CL1 that increases the ability for migration and adhesion (108, 109). Furthermore, growth factors like glial cell-derived neurotrophic factor (GDNF) and hepatocyte growth factor (HGF) have been identified as very potent chemoattractants (110, 111).

1.6.2 *In situ* Proliferation

Although macrophages that are involved in inflammation and tumor progression are known to be recruited from BMDMs, recent evidence has implied that local proliferation of resident

macrophages and microglia also contributes to the expansion during pathological conditions (109, 112).

Macrophages

Recent findings show that in an inflammatory context, such as atherosclerosis or during infection, local M2-like macrophage proliferation is a key event and leads to the expansion of this macrophage subtype (113). During nematode infection, controlled by CSF1, IL-4 drives local proliferation of macrophages indicating that proliferation is a feature of a T_H2 inflammation-oriented response (114, 115). Intriguingly, proliferation and recruitment can be interlinked as observed in adipose tissue. Here, CCL2 induces macrophage proliferation but also monocyte entry into circulation and adipose tissue (116).

Macrophage proliferation has not only been observed under inflammatory conditions but can also be found in a tumor setting. For instance, in the spontaneous MMTVneu mouse model, that resembles $HER2^+$ human breast cancer, local proliferation of fully differentiated $CD11b^{low}F4/80^{high}$ TAMs is the essential mechanism accounting for the accumulation of TAMs under CSF1R-dependent signaling. Monocyte depletion did not affect this TAM population (117). The presence of proliferating TAMs in human breast cancer material has been correlated to hormone receptor negative tumors, high grade and negative clinical outcome (118). Furthermore, we have shown that semaphorin (SEMA) 3A increases M1-like macrophage proliferation while it inhibits expansion of M2-like macrophages leading to a pro-inflammatory microenvironment that hampers tumor growth in mammary breast cancer (119). Interestingly, in a murine pancreatic ductal adenocarcinoma (PDAC) model both tissue-resident macrophages and inflammatory monocytes contribute to the local TAM population. These populations varied in the expression of cell surface markers and their function. While embryonically-derived MHC II^{low} TAMs exhibited remodeling capacities and thereby fueled PDAC progression, monocyte-derived MHC II^{high} TAMs played an important role in antigen presentation (120).

Microglia

In different brain malignancies, depending on the pathological condition, various observations have been made regarding microglia proliferation and recruitment of BMDMs. For example, acute motor nerve damage triggers microglia expansion but not an influx of monocytes (121). On the contrary, in brain tumors, both tissue-resident microglia and recruited BMDMs are present. These recruited BMDMs, upon entry, can upregulate 'microglia-specific' genes, such as *Cx3cr1*. Therefore, both populations contribute to the pool of glioma-residing myeloid cells (92, 122). However, glioma cells secrete different factors

that were shown to trigger local microglia proliferation, such as CCL2, granulocyte-macrophage (GM)-CSF, M-CSF and VEGF-A (108, 123, 124). Accordingly, M-CSF/M-CSF1R paracrine communication between microglia and GBM augmented infiltration of microglia and GBM invasion *in vivo* (125).

1.6.3. Innate-adaptive crosstalk

Macrophages and Microglia

As mentioned earlier, at the early stage of tumor initiation, M1-like TAMMs promote anti-tumor responses by secreting pro-inflammatory factors such as TNF-α and chemokines like (C-X-C motif) ligand (CXCL)9 and CXCL10. The release of these chemokines mediates activation and recruitment of cytotoxic T lymphocytes and NK cells that show anti-tumoral properties (126). However, M2-like TAMMs lose these anti-tumoral activities (52, 127, 128). They secrete immune-suppressive molecules such as IL-10 and TGF-β that down-regulate anti-tumoral cytotoxic T lymphocyte and NK cell activity (129, 130). Instead, these TAMMs stimulate the differentiation of naïve $CD4^+$ T lymphocytes into T_H2 cells and regulatory T lymphocytes that augment anti-tumoral activity (**Figure 7**) (131, 132).

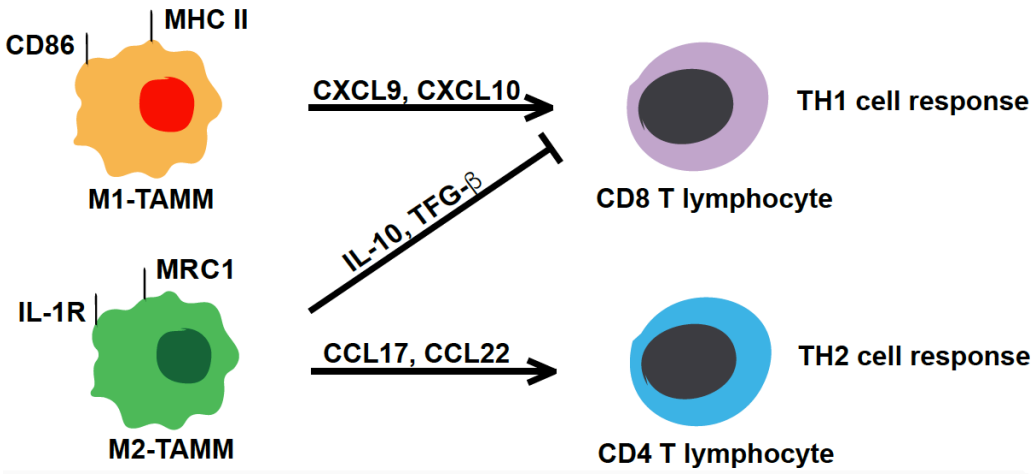

Figure 7. Cross-talk between TAMMs and T cells. M1-TAMMs activate $CD8^+$ T lymphocytes, while M2-TAMMs inhibit $CD8^+$ T lymphocytes and activate regulatory $CD4^+$ T lymphocytes.

1.6.4 Angiogenesis

Tumor angiogenesis, triggered by the angiogenic switch, induces the growth of new blood vessels from pre-existing vasculature to supply the tumor with oxygen, nutrients and is required for the tumor to grow beyond a size of 1-2 mm^3 (133-136). Hence, the balance between anti- and pro-angiogenic factors shifts toward a pro-angiogenic microenvironment (137). Many different factors regulating the angiogenic switch have been reported including VEGF-A, TGF-β and ROS (138-140). In fact, hypoxia is a key driver of angiogenesis and the secretion of VEGF-A by cancer cells and recruited leukocytes triggers the development of a vascular network (135). These tumor blood vessels are characterized by a disorganized establishment, excessive vessel branching and enlarged vessels (141, 142). The discontinuous lining of endothelial cells and pericytes but also defective basement membranes result in leaky and poorly perfused vessels that increase tumor intravasation and hamper drug delivery (143, 144).

TAMMs are key effectors in angiogenesis and are known to accumulate in poorly vascularized necrotic areas and to surround blood vessels (145). They switch into the pro-angiogenic phenotype in the presence of stress factors such as low oxygen, low pH and high lactate concentration (146). Hypoxia appears to be one of the strongest factors promoting TAMM recruitment (147, 148). Once they have reached the hypoxic sites, hypoxia changes gene expression toward pro-angiogenic genes through the modulation of hypoxia-inducible factor (HIF)-1 and HIF-2 and other transcription factors (149, 150). In response, TAMMs secrete a number of pro-angiogenic growth factors including VEGF-A, CXCL2, TNF-α and basic fibroblast growth factor (bFGF) but also matrix modulating mediators such as MMPs, urokinase-type plasminogen activator (uPA) and its receptor uPAR (87, 148, 151, 152). For instance, MMP-9 expression by microglia increases glioma growth and invasion (153). Moreover, inhibition of MMP-9 activity and expression by infiltrating macrophages reduces the release of VEGF-A from the ECM and thereby inhibits angiogenesis and tumor growth in cervical cancer (154). Interestingly, hypoxia does not seem to drive the M2-phenotype but rather fine tunes M2-like TAMMs (155). TAMs that surround blood vessels and promote angiogenesis were identified to express the Tie2 receptor (156). Accumulation of $Tie2^+$ macrophages is correlated with microvascular density and distant metastasis (**Figure 8**) (157, 158).

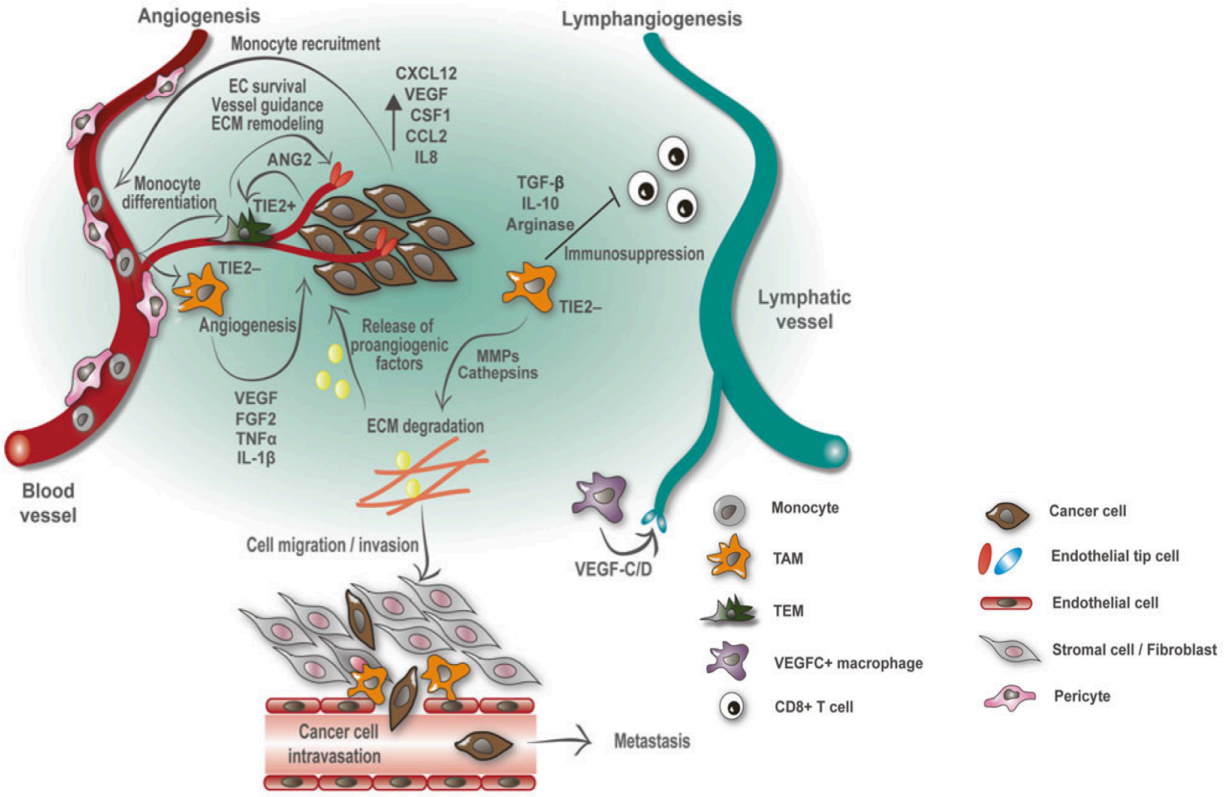

Figure 8. Modulatory functions of macrophages in the tumor microenvironment. Adapted from Macrophages: Biology and Role in the Pathology of Diseases, Chapter 7: Vascular Modulatory Functions of Macrophages. Ioanna Keklikoglou and Michele De Palma; Editors: Subhra K. Biswas and Alberto Mantovani. Springer.

1.6.5 Lymphangiogenesis

In the embryonic development lymphatic vessels are established after the cardiovascular system, when subpopulations of endothelial cells commit to the lymphatic lineage (159). Lymphatic capillaries are unidirectional and are lined with a thin single layer of lymphatic endothelial cells (LECs) anchored to the ECM (160). These LECs have a discontinuous basement membrane and lack the coverage of pericytes or smooth muscle cells that makes them highly permeable to interstitial fluids and proteins and facilitates immune cell transmigration (161, 162). Hence, the function of lymphatic vessels includes the regulation of tissue fluid homeostasis, collection of macromolecules from tissues back to the blood circulation as well as the transport of immune cells (162).

As angiogenesis, lymphangiogenesis in adult tissues only occurs during tissue repair, inflammation or tumorigenesis (163). Both VEGF-C and -D are involved in physiological

and pathological lymphangiogenesis (164, 165). Whereas the deletion of *Vegfd* in mice results in slight reduction of lymphatic vessels, deletion of *Vegfc* leads to embryonic lethality. Thus, VEGF-C is essential for initial sprouting, migration and survival of LECs (166, 167). Tumor-associated lymphatic vessel growth is positively correlated to the overexpression of VEGF-C and -D in tumors (168, 169). Tumor-associated lymphatic vessels occur mostly at the tumor margin but may also occur intratumoral (169). LECs isolated from normal tissue compared to the ones from fibrosarcoma displayed significant differences in the expression of 790 genes. Among those, genes implicated in endothelial junctions, matrix and vessel growth were upregulated, while matrix proteins like collagens and fibrillin were downregulated (170, 171).

Lymphangiogenesis is also mediated by several subpopulations including tumor cells, stromal cells, activated platelets and infiltrating macrophages (172, 173). TAMs do not only secrete the lymphangiogenic factors VEGF-C and -D, they also respond to the chemotactic properties of VEGF-C as they express its receptor VEGFR-3 (174). Thus, TAMs have been associated with lymphangiogenesis in several studies (172, 175). The accumulation of TAMs significantly correlates with lymph vessel density in several tumor tissues (176). In human breast cancer, elevated levels of VEGF-C expressing TAMs are not only associated with increased lymph vessel density but also with lymph node metastasis and lymph vessel invasion (177). Interestingly, mechanistic evidence has been provided for the involvement of TNF-α activated TAMs in lymphangiogenesis and lymphatic metastasis. These TAMs mediate the observed effects by coordinating VEGF-C/VEGFR-3 signaling (178).

1.6.6 Invasion and Metastasis

Invasion and metastasis is a multistep process that includes a cascade of different events. TAMs are one of the major players as they contribute at various steps (179). In the early stage of tumor development, macrophages are found in areas of basement membrane breakdown and in advanced tumors at the invasive front (106, 180). Tumor cells exploit the matrix deposition and remodeling capacities of TAMs that produce MMPs and other proteolytic enzymes (181, 182). For instance, the proteolytic capacity of MMP-9 releases matrix-bound VEGF-A that contributes to angiogenesis and metastasis (183, 184). Moreover, it has been shown that the release of MMPs by TAMs promotes tumor cell motility and invasiveness (148, 185-187). Thus, TAM-derived enzymes help in the establishment of a premetastatic and metastatic niche (84).

Intravasation also requires close interaction between tumor cells and TAMs. The release of CSF1 by tumor cells stimulates migration of CSF1R expressing macrophages and their production of epidermal growth factor (EGF) in turn activates invasion of EGFR expressing tumor cells. Depletion of either one of the factors of this paracrine signaling loop abrogates migration of both cell types *in vivo* (125, 188). Interestingly, Wyckoff and colleagues could show that macrophages guide tumor cells to the blood vessels and thereby increase migration and invasion (189). Additionally, TAMs increase tumor cell intravasation by contributing to vascular abnormalization creating leaky blood vessels. Thus, macrophage re-education of M2-like macrophages to M1-like macrophages inhibits endothelial cell proliferation and normalizes the vessel walls, which in turn prevents tumor cell intravasation and metastasis (190). However, at the metastatic site tumor cells recruit macrophages to also induce vessel permeability via the upregulation of VEGF-A enabling them to extravasate (191). These macrophages were characterized as $CCR2^{+}VEGFR1^{+}Ly6C^{-}F4/80^{+}$ macrophages (192).

Tumor metastasis to distant organs and lymph nodes is well established. Tumor cells can metastasize to one or more distant sites either sequentially or in parallel. (193). However, there are still contradictory studies about the role of lymph nodes in metastasis. On one hand, it is reported that lymph nodes promote further spreading and on the other hand studies point out that lymph nodes only serve as distant sites of tumor cell colonization without any further dissemination (194-197). Nevertheless, there is one common consensus about lymph node metastasis being a key prognostic marker for patient survival and outcome (198, 199).

In conclusion, TAMs play an important role in the process of tumor metastasis and high infiltration of TAMs is correlated with increased tumor cell dissemination, malignancy and tumor grade in many types of cancer (92, 145, 200).

1.7 THERAPEUTIC IMPLICATIONS

TAMs have emerged as attractive targets in anti-cancer therapy because they are the predominant cell type in many different cancers, they contribute in various ways to cancer progression and are associated with therapy resistance (73, 201, 202). Importantly, compared to cancer cells they are genetically stable and thus less susceptible to acquire therapeutic resistance (203). So far, several studies have been conducted targeting TAMs from different angels. These include inhibition of macrophage recruitment, macrophage re-education toward an anti-tumoral phenotype and suppression of macrophage survival (85, 202).

For instance, inhibition of CCL2 or VEGFR-2 reduces the infiltration of macrophages and inhibits tumor growth and angiogenesis (204, 205). Another very attractive target to block the recruitment of TAMs to tumor sites is the CSF1/CSF1R pathway. Ries and colleagues showed that blocking the CSF1R with the monoclonal antibody RG7155 reduces the recruitment of $CSF1R^{+}CD163^{+}$ macrophages to the tumor in patients suffering from diffuse-giant sarcoma improving their clinical outcome (206). Furthermore, inhibition of CSF1R reprograms microglia from a pro-tumoral phenotype to an anti-tumoral phenotype that hampers glioma development and increases survival (91). However, CSF1R inhibition as a monotherapy does not always succeed. In preclinical models of breast cancer there was only an effect in tumor growth suppression when CSF1R inhibition was used in combination with other drugs, such as paclitaxel (207, 208). This different outcome points out the important fact that the composition of anti-cancer therapy is dependent on the cancer type and location and that monotherapy is not sufficient enough as it only delays tumor growth. Therefore, the combination with irradiation, anti-angiogenic therapies, adoptive T cell transfer and immune checkpoint inhibitors represents attractive partners (209-212).

Since it is known that M1-like macrophages execute anti-tumoral activity, re-educating pro-tumoral M2-like macrophages to anti-tumoral M1-like macrophages illustrates another attractive strategy. In this respect, several studies have shown anti-cancer potential using this approach. Coscia and colleagues have shown that zoledronic acid skews macrophages from an M2-like to an M1-like phenotype and thereby inhibits spontaneous mammary carcinoma development (213). Moreover, histidine-rich glycoprotein induces the re-education of TAMs toward an M1-like phenotype resulting in vessel normalization, increased vessel functionality and hampering of tumor growth and metastasis (190).

Finally, suppression of TAM survival has also been shown to improve therapeutic outcomes. Cieslewicz and colleagues developed an M2-like macrophage specific pro-apoptotic peptide (M2pep) that selectively killed TAMs in tumor-bearing mice improving their survival rates (214).

All these different approaches display high potential of using TAMs as a target in anti-cancer therapy and underline that the microenvironment is an important player to consider in successful treatment.

2 AIMS OF THE THESIS

The overall aim of this thesis is to elucidate the diverse functions of tumor-associated macrophages and microglia in tumorigenesis and metastasis. Understanding the mechanisms of how macrophages and microglia contribute to tumor malignancies in different microenvironments helps to develop tools that specifically target distinct subpopulations.

The thesis consists of the following four studies:

Study I: Exploring the effect of SEMA3A on the accumulation of anti-tumoral macrophages and their anti-tumor activity in mouse and human breast cancer.

Study II: Elucidating the role of macrophage- and tumor-derived VEGF-C on tumor metastasis in mouse and human breast cancer.

Study III: Studying the effect of Cripto-1 vaccination on metastasis and cancer stem cells in mammary carcinoma.

Study IV: Examining the function of macrophages and microglia in PDGFB-driven mouse and human glioma.

3 RESULTS AND DISCUSSION

3.1 TUMOR MOUSE MODELS

TAMMs, major players in tumor progression and metastasis, can be of an anti- or pro-tumoral phenotype that can either restrict or fuel tumor development at various steps. In this thesis we have investigated the anti- and pro-tumoral properties but also functional differences of TAMMs in breast cancer and glioma.

In **study I-III** we used the 4T1 mammary tumor mouse model to investigate tumor-associated macrophages and tumor cells in breast cancer evolvement and dissemination. 4T1 tumor cells were originally isolated from a single spontaneous tumor that arose in a BALB/cfC3H mouse and resembles human TNBC (215, 216). We engineered the cell line according to our aims to either overexpress SEMA3A, VEGF-C or cripto-1 and injected the cells orthotopically into the mammary fat pad of mice.

There are several advantages of using 4T1 tumor cells to study breast cancer. First, it is an easy transplantable cell line that is highly tumorigenic and invasive. As it is injected into the mammary fat pad it grows in the anatomically correct site. Second, 4T1 tumor cells spontaneously metastasize from the primary tumor mainly via the hematogeneous route to lung, liver, brain, bone and blood and to a lesser extent to the lymph nodes. Thus, 4T1 tumor cell dissemination is similar to that of human breast cancer. Finally, 4T1 tumor cells are resistant to 6-thioguanine enabling the quantification of metastasis in the mentioned distant organs (217). Of note, this model displays high accumulation of granulocytes into the tumors and lungs, which is a dissimilarity to human TNBC.

In **Study IV** we expanded our knowledge about macrophages by elucidating the functional role of microglia and their difference to macrophages in glioma development. We used the RCAS/*tv-a* mouse glioma model. Mice of this model harbor two genetic aberrations including the systemic depletion of the tumor suppressor Arf and the expression of the *tv-a* gene in glial progenitor cells that contain the nestin promoter. The *tv-a* gene encodes the receptor for the avian leukosis virus (ALV) and is normally only expressed in avian cells. The viral vector RCAS that is derived from the same virus is packaged with *pdgfb* and newborn mice are transduced with this virus allowing the selective transduction of glial progenitor cells that harbor the nestin promoter. Thus, those mice develop PDGFB driven N/tv-a;Arf$^{-/-}$ gliomas at high rates up to 100% incidence at 12 weeks of age (218, 219). This glioma model

is a well established and widely used model to study the mechanisms of glioma development and provides a life-like model of human glioma of grade II to IV (219).

3.2 STUDY I

Guidance molecule SEMA3A restricts tumor growth by differentially regulating the proliferation of tumor-associated macrophages

Semaphorin (SEMA)3A is a secreted protein that was originally discovered as an axon guidance regulator in establishing the neuronal network in the embryonic nervous system (220, 221). This protein is found in most human tissues and binds to its co-receptor neuropilin (NP)1 that forms receptor complexes with receptors of the Plexin family (222). However, in the last decade SEMA3A has been implicated in cancer development. Except for pancreatic cancer, where SEMA3A expression has been positively correlated to increased malignancy grade, this protein is down-regulated in several human cancers such as breast, gastric, epithelial ovarian and non-small cell lung cancer (119, 223-226).

Animal studies investigating the effect of SEMA3A on macrophages in the tumor microenvironment show contradictory results. Carrer and colleagues demonstrated that SEMA3A promotes vessel maturation through the recruitment of circulating NP1 expressing monocytes. These $CD11b^{+}NP1^{+}Ly6G^{-}$ monocytes, isolated either from the bone marrow or from SEMA3A-expressing muscles and directly injected into growing tumors, exerted anti-tumoral activity by induction of vessel normalization and inhibition of tumor growth (227). On the other hand, Casazza and colleagues reported that SEMA3A is induced in hypoxic areas and acts as an attractant for NP1-expressing TAMs. Once those TAMs have arrived in the hypoxic environment, they are entrapped inside the hypoxic niche and exert pro-angiogenic and immune-suppressive functions promoting tumor growth and metastasis (228).

However, knowing that SEMA3A expression is down-regulated in many different human cancer types, we also checked SEMA3A expression levels in different grades of human breast cancer samples. In concordance, we found a down-regulation of SEMA3A with disease progression. To further study the effect of SEMA3A on tumor development, we used the 4T1 tumor mouse model with 4T1 cells that overexpressed SEMA3A. Tumors overexpressing SEMA3A showed reduced tumor growth and burden. When we dissected the tumors and looked at the immune cell composition, we discovered an increased accumulation

of anti-tumoral M1-like macrophages and cytotoxic lymphocytes including T lymphocytes and NK cells.

The M1- and M2-like phenotypes of macrophages were determined by the expression of different cell surface markers using flow cytometry and gene expression profiles via RT-qPCR. Using flow cytometry we classified macrophages as $CD11b^{+}Ly6G^{-}$ cells. On the basis of MHC class II expression we further subdivided macrophages into $Ly6C^{lo}$ MHC class II^{hi} M1-like macrophages and $Ly6C^{lo}$ MHC class II^{lo} M2-like macrophages. Additionally, we further verified our observation by investigating M1- and M2-markers that are involved in costimulation, antigen presentation and scavenging including CD11c, CD80, CD86, MHC class I and MRC1 in $CD11b^{+}F4/80^{+}$ macrophages. Sorted $CD11b^{+}F4/80^{+}$ macrophages from SEMA3A and CTR tumors were subjected to RT-qPCR looking at a range of cytokines and growth factors confirming our result of increased M1-like macrophage accumulation in SEMA3A tumors.

Based on our results and the fact that tumors are infiltrated by monocytes, we speculated that monocyte recruitment might account for the observed effect. However, there was no increase in monocyte frequency in the blood or tumor in SEMA3A overexpressing tumors. Furthermore, direct administration of SEMA3A to BMDMs did not affect their phenotype. Interestingly, publications about local macrophage proliferation led us to investigate if SEMA3A affected proliferation rather than recruitment of M1- and M2-like macrophages. Indeed, we could show both *ex vivo* and *in vivo* that SEMA3A mediates the expansion of M1-like macrophages, while it reduces the proliferation of M2-like macrophages. This effect was NP1 dependent and regulated Akt and MAPK signaling. Intriguingly, knockdown of NP1 in BMDMs mimicked the effect of SEMA3A on Akt and MAPK phosphorylation mediated by CSF1 stimulation. Thus, CSF1 stimulated M1-BMDMs showed increased Akt and MAPK phosphorylation after SEMA3A treatment or NP1 inhibition, while M2-BMDMs displayed reduced Akt and MAPK phosphorylation. NP1 can undergo signaling complexes with various members of the Plexin A family and therefore, we speculate that differential expression of Plexin A receptors and diverse dimerization complexes with NP1 could induce the opposing outcome in M1- and M2-macrophage proliferation. Further investigations are needed to explore the detailed mechanism of SEMA3A-mediated macrophage proliferation.

However, SEMA3A overexpressing tumors did not only show accumulation of anti-tumoral macrophages but also of cytotoxic $CD8^{+}$ T lymphocytes and NK cells. The increased recruitment and activation of $CD8^{+}$ T lymphocytes and NK cells was dependent on and mediated by SEMA3A via NP1 expression on macrophages. Nevertheless, the observed

reduced tumor growth of SEMA3A tumors was attributed to both macrophages and cytotoxic cells. Accordingly, upon depletion of the different populations SEMA3A lost its effect on tumor growth inhibition.

In sum, we show that SEMA3A mediates the proliferation of M1-like macrophages that creates a more pro-inflammatory microenvironment enhancing the recruitment and activation of cytotoxic $CD8^+$ T lymphocytes and NK cells and thereby inhibiting breast cancer progression (**Figure 9**).

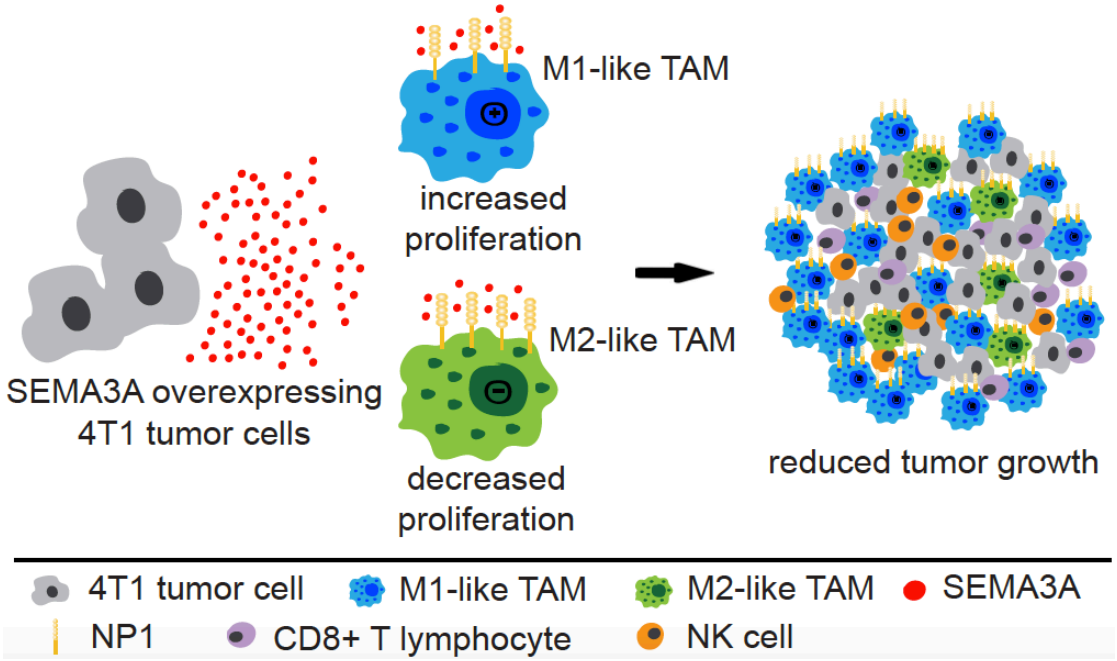

Figure 9. SEMA3A enhances the expansion of M1-like macrophages and restricts the proliferation of M2-like macrophages. The selectively regulated proliferation recruits and activates cytotoxic $CD8^+$ T lymphocytes and NK cells and thereby inhibits tumor progression.

3.3 STUDY II

Macrophage-derived Vascular Endothelial Growth Factor-C decreases hematogeneous metastatic dissemination by normalizing the tumor vasculature

VEGF-C is a secreted dimeric glycoprotein and one of five family members in mammals (229). In adults VEGF-C expression is found in many different organs including heart, lung, pancreas and kidneys (230, 231). VEGF-C can bind to its two receptors VEGFR-2 that interacts with the co-receptor NP1 and VEGFR-3 that binds NP2 (230, 232, 233). The affinity of VEGF-C to either one of the receptors depends on the proteolytic cleavage. While non-processed VEGF-C activates VEGFR-3 signaling, mature VEGF-C binds with high

affinity to both VEGFR-2 and -3 and can induce the formation and activation of receptor heterodimers (**Figure 10**). These heterodimers have been reported to be present on lymphatic vessels but also on angiogenic sprouts pointing out a bimodal function for VEGF-C and its receptors (234-236). In fact, a study conducted by Tammela and colleagues showed that $Tie2^{+}VEGF\text{-}C^{+}$-macrophages accumulate behind tip cells at vascular branching points inducing endothelial cell differentiation via VEGFR-3 and Notch signaling. Thus, this finding indicates a function of VEGF-C-expressing macrophages in vessel maturation (237). Of note, processing of VEGF-C also affects its binding to the two co-receptors NP1 and NP2. While the partially processed protein binds to both NP1 and NP2, only the fully mature protein can bind NP2 showing another layer of complexity in VEGF signaling (238).

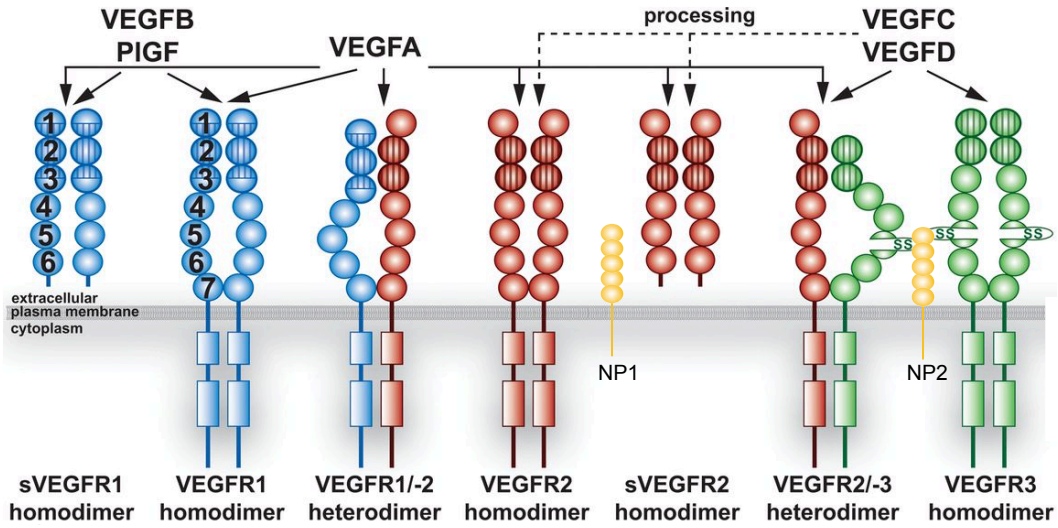

Figure 10. VEGF-C binding specificities and VEGFR signaling complexes. Adapted and modified from (239).

However, VEGF-C expression has been detected in several different types of human tumors including breast, cervix, colon, lung, prostate and stomach (240-242). Although VEGF-C expression is predominantly correlated to lymphangiogenesis, it has been shown that tumor cell-secreted VEGF-C binds to VEGFR-3 that is upregulated on angiogenic blood vessels in breast cancer (243-245). Several different types of tumors express VEGF-C but TAMs have been reported to be a major source of this protein and as mentioned before they correlate to lymphatic microvessel density, lymphangiogenesis, invasion and lymph node metastasis in several types of cancer (172, 174, 177, 246, 247).

As tumor dissemination occurs later during cancer progression and macrophages are active participants in this process, it would be natural to assume that macrophages and tumor cells secrete increased levels of VEGF-C leading to lymph node metastasis in advanced breast cancer. However, when we looked at VEGF-C expression in ER^+ grade I and III ductal carcinoma and grade III TNBC, we found unexpectedly decreased VEGF-C expression by $CD68^+$ macrophages in grade III compared to grade I breast cancer. Moreover, the overall expression of VEGF-C within the tumor seemed to be similar throughout the different grades. This evidence prompted us to further investigate if tumor- and macrophage-derived VEGF-C might be differentially regulated during tumor progression and thereby influence the route of tumor cell dissemination.

To investigate our hypothesis we chose to use the 4T1 tumor mouse model. As mentioned earlier this mouse model resembles TNBC and mainly metastasizes via the hematogeneous and not lymphangiogenic route. Accordingly, we expected high levels of VEGF-A in macrophages and tumor cells. Therefore, macrophages and tumor cells were sorted from 4T1 tumors and VEGF-A, -C and -D expression levels were analyzed by RT-qPCR. Intriguingly, macrophages only expressed VEGF-A while VEGF-C and -D were undetected. However, tumor cells expressed both VEGF-A and VEGF-C to a similar extent. Nevertheless, it was evident that macrophages expressed 10 times higher levels of VEGF-A compared to tumor cells, so that we speculated that macrophage-derived VEGF-A rather than tumor-derived VEGF-A dictates the preferred hematogeneous route for 4T1 cancer cell dissemination. We further hypothesized that if we engineer macrophages to overexpress VEGF-C, we might be able to change the preferable route of dissemination from blood vessels to lymphatic vessels.

To test our theory, we engineered chimeric mice that had been transplanted with bone marrow-derived stem cells to express VEGF-C using lentiviral-mediated gene transduction. These chimeric mice were then inoculated with 4T1 tumor cells. In comparison, to test what effect tumor-derived VEGF-C has, 4T1 tumor cells were also engineered to overexpress VEGF-C by lentiviral-mediated gene transduction and injected into the fat pad of naïve mice. Neither macrophage- nor tumor-derived VEGF-C altered tumor growth or burden compared to their respective controls. However, tumor- and macrophage-derived VEGF-C had a different effect on lung metastasis. While both tumor- and macrophage-derived VEGF-C increased lymph node metastasis, only macrophage-derived but not tumor-derived VEGF-C decreased lung metastasis. In fact, tumor-derived VEGF-C increased pulmonary metastasis but not as a consequence of increased angiogenesis. Consequently, we considered another important factor contributing to tumor spreading, namely vessel dysfunctionality. Therefore,

we determined blood vessel perfusion, pericyte coverage and hypoxia. Interestingly, macrophage-derived VEGF-C increased vessel perfusion, pericyte coverage and decreased hypoxia while tumor-derived VEGF-C had the opposite effect. Improved vessel functionality is attributed to the M1-like phenotype but VEGF-C expression in macrophages or tumor cells did not shift the macrophage phenotype toward an M1- or M2-like phenotype. Moreover, neither the infiltration of cytotoxic $CD8^+$ T lymphocytes nor NK cells was altered. Further investigations are necessary to elucidate the exact mechanism of improved vessel functionality by macrophage-derived VEGF-C. It would be interesting to elaborate if processing of VEGF-C and the binding to its two receptors VEGFR-2 and -3 is regulated differentially by tumor cells and macrophages leading to the various effects on vessel functionality.

Taking all results in perspective, we show that macrophage-derived VEGF-C normalizes the tumor vasculature and thereby decreases pulmonary metastasis while tumor-derived VEGF-C increases vessel abnormalization and lung metastasis (**Figure 11**).

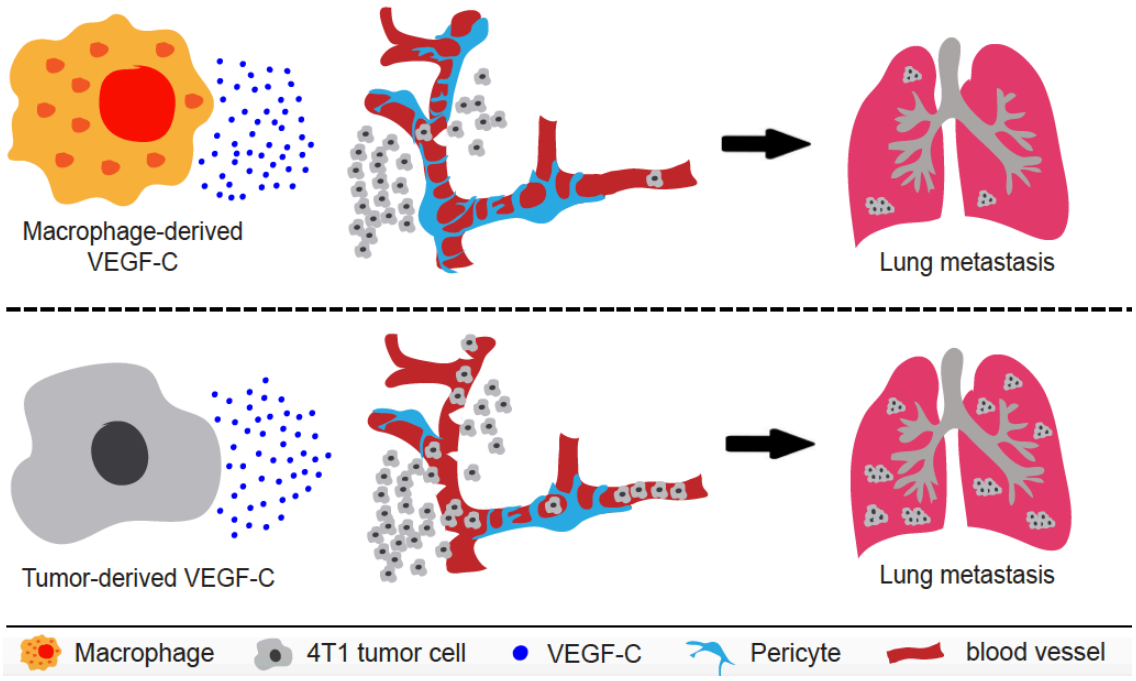

Figure 11. Macrophage-derived VEGF-C normalizes the blood vessels and thereby decreases lung metastasis, while tumor-derived VEGF-C decreases vessel functionality leading to increased pulmonary metastasis.

3.4 STUDY III

Cripto-1 plasmid DNA vaccination targets metastasis and cancer stem cells in murine mammary carcinoma

Cripto-1 is a protein that plays an important role during embryonic development contributing to the undifferentiated status of human and mouse embryonic stem cells (248). However, more than 50% of human cancers, including breast cancer, show elevated Cripto-1 expression levels and its presence on cancer cells has been associated with the cancer stem cell (CSC) phenotype (249, 250). CSCs are a subpopulation that initiates tumor growth, shows enhanced metastatic potential, is resistant to radiation and chemotherapy and responsible for relapse (249, 251). Indeed, cripto-1 has been shown to be involved in proliferation, angiogenesis and invasion and high levels are associated with colorectal metastasis and poor prognosis in colon and breast cancer (252-256). Therefore, Cripto-1 displays an interesting candidate to specifically target metastatic cancer cells. For instance, in a murine melanoma model Cripto-1 vaccination elicited a specific cytotoxic $CD8^+$ T lymphocyte mediated protective response by reducing primary tumor burden and lung metastasis (257).

Hence, we wanted to investigate if Cripto-1 vaccination also had a protective effect in mammary breast cancer progression and metastasis. Therefore, different cell lines were checked for Cripto-1 expression. Cripto-1 could be detected in the highly metastatic 4T1 tumor cell line and in TUBO cells that show a cancer stem cell like phenotype. However, Cripto-1 expression in 4T1 cells was very low and not detectable via flow cytometry, so that we decided to generate a stable Cripto-1 expressing 4T1 cell line that could be used in our *in vivo* studies. Before inoculation of these cells into the mammary fat pad, mice were vaccinated with Cripto-1 encoding DNA plasmids. Intriguingly, Cripto-1 vaccination did not only reduce tumor growth and burden but also decreased lung metastasis.

We then wondered how this effect was mediated. Vaccination goes along with cell- and antibody-mediated immunity. Accordingly, we speculated that Cripto-1 vaccination could evoke a humoral response in mice. Indeed, we detected Cripto-1 specific antibodies in serum of vaccinated mice that could bind to Cripto-1 expressing 4T1 cells. Moreover, most of the antibodies belonged to the subclasses IgG2a and IgG2b that in mice mediate antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells. To test if Cripto-1 specific antibodies induce ADCC, we investigated if serum from Cripto-1 vaccinated mice could increase NK cell cytotoxicity. In fact, pre-activated NK cells showed increased lysis of

Cripto-1 expressing 4T1 cells in the presence of serum from Cripto-1 vaccinated mice *in vitro*.

Furthermore, to verify our observations of reduced tumor and metastatic burden in our validation model (Cripto-1 expressing 4T1 tumor mouse model), we used the more clinical relevant BALB-neuT mouse model. Intriguingly, despite Cripto-1 negative primary tumors, we observed a protective effect of Cripto-1 vaccination on the metastatic burden in the lungs. Furthermore, Cripto-1 vaccination effectively decreased tumor growth of TUBO cells that show CSC characteristics and even led to tumor clearance, as three out of eleven mice stayed tumor free for more than 60 days.

Taking all together, Cripto-1 vaccination induced a humoral response facilitating NK cell-mediated ADCC and the reduction of lung metastasis (**Figure 12**).

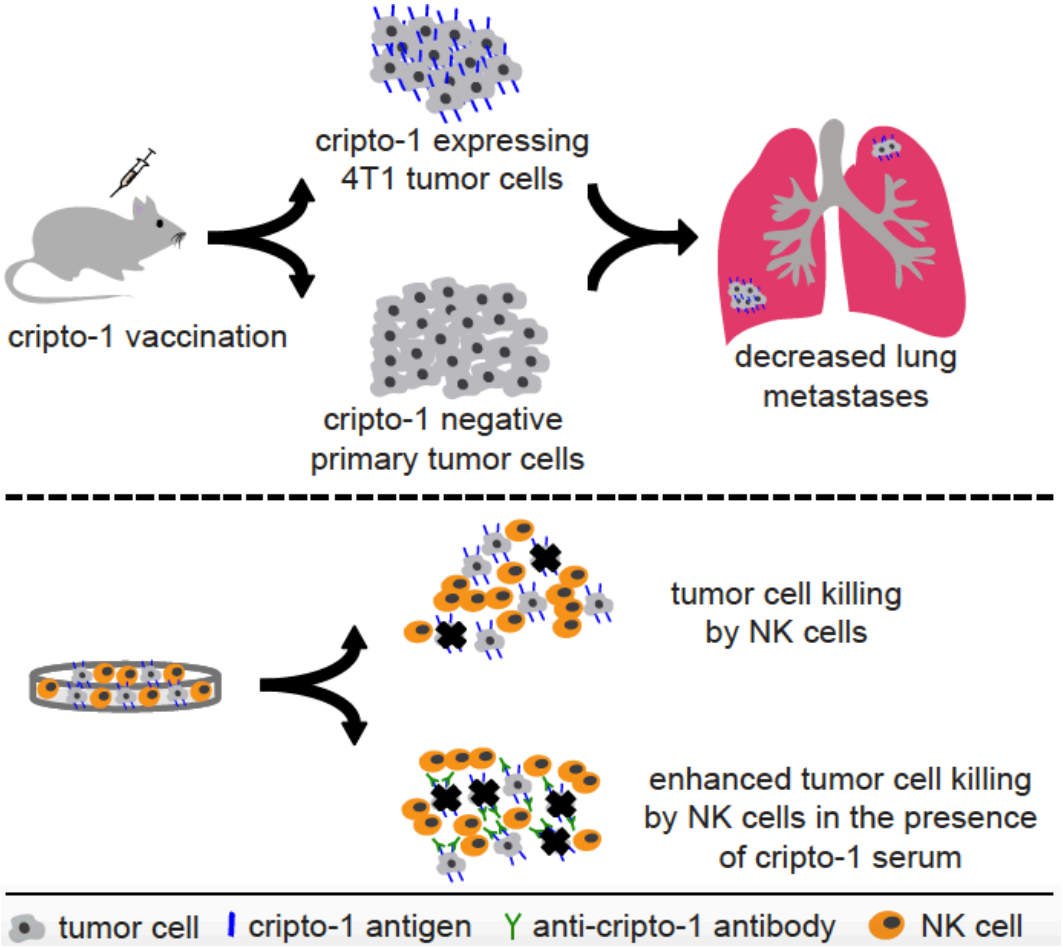

Figure 12. Cripto-1 DNA plasmid vaccination decreases the metastatic burden in lungs of mice harboring Cripto-1 positive and negative primary tumors. NK cells show enhanced killing of Cripto-1 expressing 4T1 tumor cells in the presence of Cripto-1 serum.

3.5 STUDY IV

Microglia induce PDGFRB expression in glioma cells to enhance their migratory capacity

Platelet-derived growth factor (PDGF)B and one of its receptors, PDGFRB, are essential for physiological processes like proliferation, migration and blood vessel development but have also been linked to pathological conditions, such as glioma development (258-260). This signaling pathway is mostly linked to tumor-associated vasculature and angiogenesis. For instance, PDGFB expression by endothelial cells regulates vessel permeability and vascular maturation in extracranial solid tumors (260, 261). Moreover, tumor cells that overexpress PDGFB induce displacement of $PDGFRB^+$ pericytes from endothelial cells, which increases vessel dysfunctionality and metastatic dissemination (262). However, even though not one of the most prevalent alterations, aberrations in PDGFB expression and/or PDGFRB phosphorylation are observed in gliomas and GBMs (218, 260, 263). Interestingly, there is one study reporting that PDGFRB is commonly expressed in cultured patient-derived GBM cells, especially by self-renewing GBM stem cells (264).

At the time when this study was initiated PDGFB-driven glioma was not well characterized with regards to the tumor and microenvironmental signature. Using the RCAS/*tv-a* mouse glioma model we defined PDGFB driven N/tv-a;$Arf^{-/-}$ gliomas and elucidated the functional properties of microglia and macrophages.

In collaboration with a neuropathologist, according to histopathological characteristics of human gliomas, PDGFB driven N/tv-a;$Arf^{-/-}$ mouse gliomas were divided into grade II- to grade IV-like gliomas, where grade IV-like gliomas showed features of human GBMs. Moreover, these low- and high-grade gliomas could be identified as astrocytomas based on their expression of GFAP.

We then continued to elaborate on vessel functionality and analyzed vessel perfusion and hypoxia. With increased grade of malignancy the number of perfused vessels was significantly decreased while hypoxia was markedly increased. Since pericyte coverage of blood vessels is important for vessel maturation and function, we assumed that high-grade gliomas have decreased pericyte coverage and immunostained the gliomas with the pericyte marker α-smooth muscle actin (α-SMA). Surprisingly, α-SMA^+ cells accumulated with increased malignancy and these cells were rarely found in conjunction with vessels but instead spread throughout the tumors. This finding prompted us to further investigate if those α-SMA^+ cells were actually pericytes. Therefore, we checked different mesenchymal/pericyte

and tumor cell markers and could identify those cells as a subpopulation of tumor cells that expressed α-SMA and PDGFRB.

Considering that α-SMA$^+$ and PDGFRB$^+$ glioma cells were scattered throughout the tumor mass, we wondered if these cells were associated to other types of stromal cells, such as TAMMs. Indeed, we found IBA1$^+$ TAMMs in close proximity to α-SMA$^+$ and PDGFRB$^+$ glioma cells. We then continued investigating PDGFRB expression on glioma cells and their co-localization with TAMMs. In concordance, we found increased PDGFRB expression on glioma cells in high-grade gliomas compared to low-grade gliomas. Along with this finding we observed increased IBA1$^+$ TAMM accumulation that could be correlated to PDGFRB expression on glioma cells. Between 50-65% of those glioma cells were in direct physical contact to IBA1$^+$ TAMMs. However, we were curious about how relevant our observations in mouse astrocytomas would be for human astrocytomas. Hence, we looked at PDGFRB expression on glioma cells and TAMM accumulation in grade II to IV human astrocytomas. In fact, we could verify the correlative accumulation of TAMMs and increased PDGFRB expression on glioma cells throughout the grades of human astrocytomas (**Figure 13**).

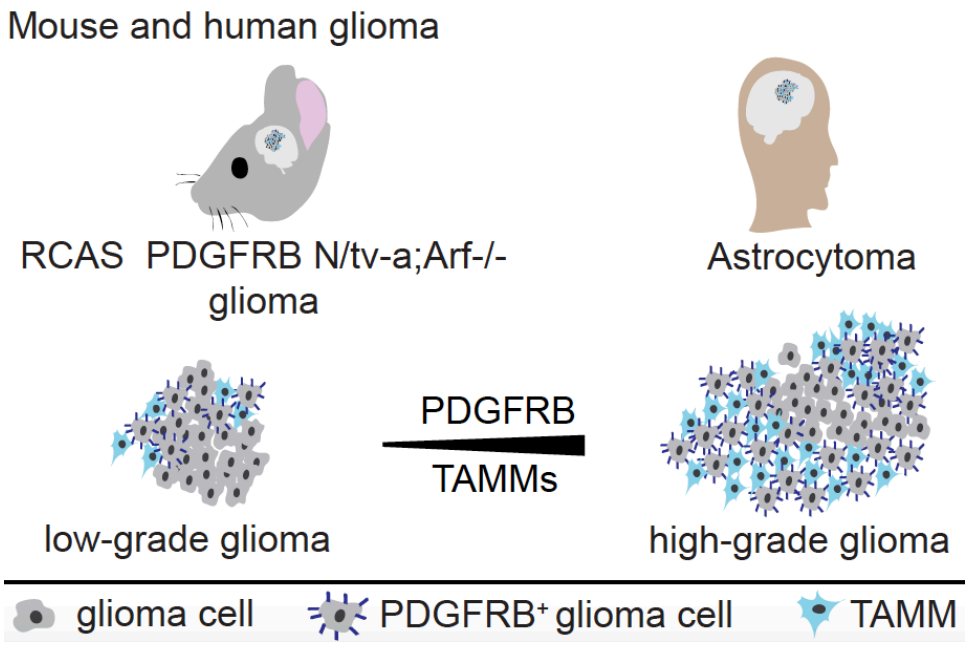

Figure 13. Mouse and human astrocytomas show increased expression of PDGFRB on glioma cells with increased grade of malignancy, which correlates to the accumulation of TAMMs.

Based on these results, we hypothesized that TAMMs could induce PDGFRB expression on glioma cells. As mentioned earlier, under physiological conditions, microglia are the abundant cell type in the brain. However, when pathological malignancies occur, the blood brain barrier is disrupted and monocytes can infiltrate the tumor. Therefore, we performed co-culture experiments with glioma stem cells together with either microglia or BMDMs that were polarized to an M1-like phenotype (LPS+IFNγ) or M2-like phenotype (IL-4+TGF-β+IL-10) *ex vivo*. To our surprise, only cell-to-cell contact with microglia but not BMDMs could induce glioma cells to express PDGFRB. Moreover, even though both M1- and M2-like microglia could induce PDGFRB expression, M2-like microglia induced significantly higher expression levels of this receptor. Importantly, only M2-like microglia were able to increase the migratory capacity of glioma cells via the upregulation of PDGFRB. Of note, BMDMs that have been pre-conditioned with microglia-derived factors also failed to induce PDGFRB expression on glioma stem cells. In conclusion, microglia but not BMDMs induce PDGFRB expression on glioma cells and thereby enhance their migratory capacity (**Figure 14**).

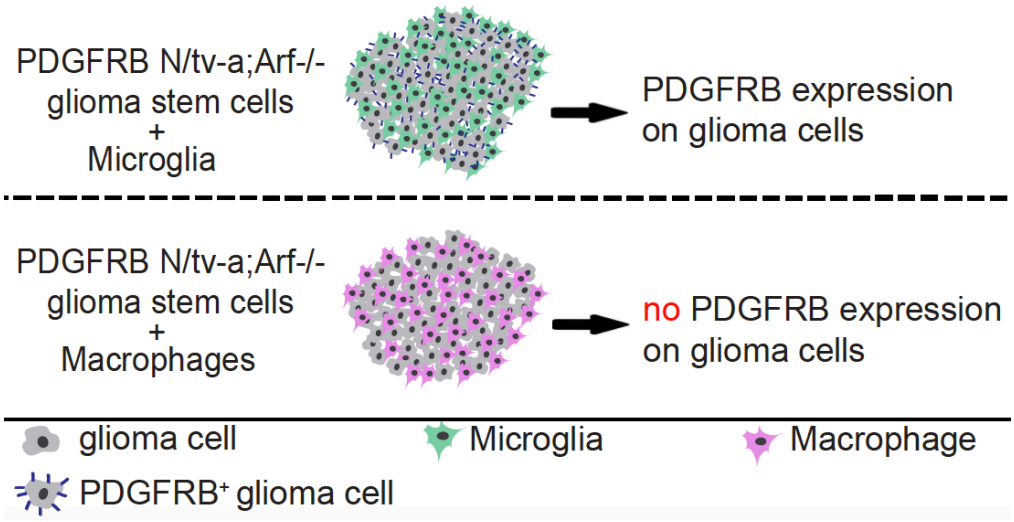

Figure 14. Microglia induce PDGFRB expression on glioma cells and thereby increase their migratory capacity. Macrophages fail to induce PDGFRB expression on glioma cells.

The finding that BMDMs failed to induce PDGFRB expression on glioma cells *in vitro*, sheds more light on the functional differences between microglia and macrophages that have not been fully discovered yet. This is especially important in therapy treatment because approaching these two subpopulations differentially might present new possibilities in glioma treatment.

4 CONCLUSIONS

Cancer is a complex disease that involves the immune system at every step of its growth and spread to secondary organs. The wide variety of mutations and differential microenvironmental signatures that may be encountered throughout the various cancer types make it very difficult to successfully treat the disease. Macrophages and microglia are abundant cells in all types of cancers. Whether these cells are allies or enemies to cancer depends on their microenvironmental surroundings and stimuli. In this thesis we have demonstrated the diverse functions of macrophages and microglia in cancer evolvement and invasion, and have shown that modulating, rather than depleting distinct subpopulations of macrophages and microglia, offers more opportunities to develop successful strategies in anti-cancer therapies. Moreover, we have illustrated that there are functional dissimilarities between macrophages and microglia. These dissimilarities provide evidence that these two populations not only differ ontologically but also behave differentially when interacting with tumor cells. These findings may facilitate better understanding of the efficiency and outcome of therapy treatments.

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